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Inventor: EPHRAIM SAUL ANDERSON

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COMPLETE SPECIFICATION

Improvements relating to Typhoid and Paratyphoid Vaccines

We, NATIONAL RESEARCH DEVELOPMENT Corporation, a Corporation established by Statute, of Kingsgate House, 66—74 Victoria Street, London, S.W.1, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to anti-typhoid vaccines and anti-paratyphoid vaccines and to the selection and culture of organisms for use in the manufacture of such vaccines. The invention is of primary importance for and will for convenience be described mainly in relation to anti-typhoid vaccines. It is, however, to be understood that where the context permits the ensuing description is to apply also to vaccines for protection against paratyphoid A and B infections.

In the manufacture of anti-typhoid vaccines it is standard practice to employ *Salmonella typhi*, an organism which has three major antigenic components, described respectively as the O, Vi and H antigens (also designated TO, TVi and TH antigens, the letter T signifying typhoid). The TH antigen is constituted by the flagella, which are attached to the body of the organism, and are responsible for its motility, and the antibodies produced by this antigen are correspondingly known as TH antibodies. There is no evidence that the TH antibodies protect against infection, but they are often the first to be produced in the body in the natural disease of typhoid fever and, because of this, the detection of TH agglutinin is a valuable diagnostic test for the disease. Inoculation with the traditional vaccine also results in the appearance of TH antibodies, which may persist indefinitely. Because of this, the diagnostic value of the test for TH antibodies is lost in persons who have previously been inoculated with the vaccines in current use. In spite of this defect, which derives from the use of the traditional vaccine, no effective solution to the problem has hitherto been proposed.

According to the present invention, a killed anti-typhoid or anti-paratyphoid vaccine formulated for parenteral administration to humans comprises, as the protective antigen, a stable non-motile strain of *Salmonella typhi* or *Salmonella paratyphi* (A or B) which is devoid of flagella capable of producing the corresponding TH, AH or BH antibodies. As the O and Vi antigens are believed to be important in the production of human immunity to typhoid fever the *S. typhi* organisms used for preparing the vaccine most desirably contain an adequate complement of O and Vi antigens.

Suitable strains of organism for the purpose of this invention may be obtained by a variety of methods. It is most important to ascertain by rigorous selection that the non-motile strain employed according to the invention is stable, i.e. does not back-mutate to the motile form. Naturally occurring non-motile strains of the organism may be encountered on rare occasions; these may be otherwise antigenically complete. Alternatively, techniques may be employed for the selection of mutant strains which are genetically incapable of producing flagella, and one very fruitful method of selection is the application of a bacteriophage which attacks only motile organisms, to a culture of the motile strain which is traditionally employed for vaccine production, or to any other motile strain of the species. The selective action of the bacteriphage reduces the proportion of motile bacteria and the genetically flagella-free mutants are thus able to emerge.

Strains may be freed from flagella by physical means such as mechanical removal, or by growth of the organism at temperatures unsuitable for flagellar formation. Alternatively, growth of organisms in media in which the formation of flagella is inhibited either by the lack of a substance essential for their synthesis, such as a particular amino acid,

[*Price 4s. 6d.*]

or by the presence of an inhibitory agent such as phenol, may result in the production of organisms devoid of flagella. Organisms freed from flagella by the means described in this paragraph will produce their usual flagella if allowed to grow under normal conditions. They are thus devoid of flagella phenotypically, rather than genotypically as in the case of true non-motile mutants, but provided that vaccine is produced from them in a medium in which the non-mutile phenotype is preserved, the vaccine will be free of the disadvantage of the conventional vaccine. It will be appreciated that separate vaccines for protection against typhoid, paratyphoid A and paratyphoid B, infections respectively, may be prepared by the methods described herein. However, as with the traditional combined vaccine which confers protection against all three organisms (TAB vaccine), a combined vaccine in which at least one of the protective antigens has the properties described above may also be produced. Thus, a vaccine prepared from an artificially selected genetically non-motile strain of *S.typhi* and non-motile strains of *S.paratyphi* A and *S.paratyphi* B will in practice be a preferred embodiment of the invention.

The vaccine will also preferably contain an independent antigenic marker, designed to provide a convenient means of determining whether a patient has been inoculated with the new vaccine. Such a marker will be an uncommon antigen and may even be derived from flagella provided they are not of the kind to produce TH, AH or BH antibodies as the case may be. Usually it will be convenient to prepare the marker separately and incorporate it at a suitable concentration in the vaccine. Rare flagellar antigens which may be used as markers include, for example, killed *S. aderike* strain 680, *S. weslaco* strain 221, *S. guinea* strain 1082, and *S. irigny* strain 387, the latter having given an excellent titre of H-antibody.

It will be appreciated that after selection of suitable strains of organism the latter are cultivated to produce vaccine. The methods adopted may follow closely the traditional methods of manufacture employed with the ancestral strains or conventional strains, both as to the cultivation steps and subsequent processing into freeze-dried or liquid vaccine form. For example, heat-killed phenolized, acetone-killed and dried, and alcohol-killed and dried vaccines may be prepared.

For use in man, vaccines prepared according to this invention are administered in the same way as traditional vaccines, i.e. by subcutaneous or intradermal routes.

Strains which have been developed or chosen for the purpose of the invention include the following strains which are typical and which have been deposited in officially recognised culture collections such as the National Collection of Type Cultures, Colindale (N.C.T.C.) and the American Type Culture Collection (A.T.C.C.)

S. typhi T.N.M.I. N.C.T.C. 10404
S. paratyphi A. strain 17689 N.C.T.C. 10405
S. paratyphi B. strain BB 6981 N.C.T.C. 10406
S. irigny N.C.T.C. 10407

The invention is illustrated in the following Examples in which the percentages are by weight unless otherwise stated.

Example 1

(a) The parent bacterial strain used as the starting point is the classical vaccine strain *Salmonella typhi* Ty2, in the form of a freeze-dried culture prepared by the National Collection of Type Cultures, Colindale (ref. No. N.C.T.C. 8385).

The organism is grown in Difco broth (Difco is a Registered Trade Mark) having the following composition:—

Bacto dehydrated broth (Difco Laboratories)	20 g.
NaCl	8.5 g.
Distilled water	1000 ml.

in the presence of the anti-flagellar χ bacteriophage of Sertic and Boulgakov (Sertic, V. & Boulgakov, N.A. (1936) C. R. *Soc. Biol., Paris, 123,* 887) and serial transfers of the mixture are carried out.

Ten ml. volumes of Difco broth, prewarmed to 37°C, are used, and the starting inocula are about 10^8 organisms/ml of Ty2 and 10^9 particles/ml of χ phage. The mixtures are grown with agitation for 4 hr. at 37°. The organisms are then counted by spread-plate titrations and, after centrifugation to deposit the bacteria, the phage titre is estimated by spot titrations on type A of *S. typhi*, which is a satisfactory indicator strain. In addition, 0.1 ml of the phage-organism mixture is transferred to 10 ml of fresh broth at 37°C. At each stage a 2 ml sample of the mixture is stored in the refrigerator at 4°C for reference purposes. The phage titre in this series rises from 10^9/ml in the tube inoculated originally to about 5×10^9/ml, and stays approximately constant at that level. Colonies that are sufficiently discrete on the plates seeded with the higher dilutions in the bacterial counts are tested with TH antiserum, and those that do not agglutinate are tested with TVi antiserum. TH-negative lines that are Vi-positive are selected for further tests. All lines are repeatedly tested for sensitivity to χ phage, and any that show lysis are discarded.

The colonies are purified by serial platings, a minimum of 2 plating from the original pick being carried out, with selection of a single colony on each occasion. Slide tests are again carried out with TH antiserum and, if these

yield negative results, the colonies are subcultured to Difco broth and inoculated on to plates of motility medium (Edwards, P. R. & Ewing, W. H. (1955) Minnesota: *Identification of Enterobacteriaceae*. Minneapolis, Burgess Publishing Company). Any lines showing "flares" indicative of back-mutation to motility are discarded.

Additional control of the motility state of cultures is carried out by phase-contrast and dark ground microscopy. In this way those lines that pass the agglutination tests but nevertheless have a small number of motile organism are detected and discarded.

Usually, many hundreds of single-colony lines require to be tested in this way before isolation of a colony which satisfies criteria for a non-motile mutant; viz. . . . it does not agglutinate with TH antiserum on slide or in tube; it does not produce flares in repeated tests in motility medium; no motile organisms are detected in phase-contrast or dark-ground microscopic examination; it is resistant to χ phage. One mutant which has been produced as described has been designated TNM1. It is Vi-positive and reacts as Vi-phage type B1 in the same way as the ancestral Ty2.

It does not agglutinate with TH anti-serum and its Vi and O agglutinability are closely similar to those of the parent strain Ty2.

(b) *Growth and harvesting of bacteria*

1 ml. of a fully grown nutrient broth (Difco broth) culture of the organism is inoculated into each of 2 × 200 ml. medicine flats containing 50 ml. of nutrient agar, and incubated at 38.5°C for 16 hr. The growth in the two bottles is washed off in a total volume of 20 ml. 0.85% NaCl. The suspension is centrifuged at 3000 r.p.m. for 30 min. and the supernatant removed. The deposit is resuspended in 0.85% NaCl to give a concentration of 4.0×10^{10} organisms/ml. It is centrifuged at 500 r.p.m. for 1 min. to remove coarse particles and then treated as follows, sufficient extra liquid being added in each treatment to reduce the concentration of organisms to 4.0×10^9/ml.

(c) *Subsequent processing*

(1) *Heat killed, phenolized* (TNM1PL)
18 ml. of 0.85% NaCl is added to 2 ml. of concentrated bacterial suspension. The mixture is thoroughly shaken and then heated at 57°C for 40 min. When it has cooled, phenol is added to a final concentration of 0.5%. It is subsequently left at ambient temperature for 48 hr. after which it is tested for sterility and transferred in 0.25 ml. quantities to ampoules which are sealed and stored at 4°C.

(2) *Acetone-killed and dried* (TNM1A)
18 ml. of acetone is added to 2 ml. of concentrated bacterial suspension. The mixture is thoroughly shaken and left at ambient temperature for 48 hr. after which it is tested for sterility and transferred in 0.25 ml. quantities to ampoules. The ampoules are dried in a desiccator at ambient temperature at a pressure below 10 mm. of mercury over concentrated sulphuric acid and then sealed. They are stored at 4°C.

(3) *Alcohol-killed and dried* (TNM1B)
3 ml. of 0.85% NaCl and 15 ml. of absolute ethyl alcohol are added to 2 ml. of concentrated bacterial suspension. The final alcohol concentration is 75%. The mixture is thoroughly shaken and left at ambient temperature for 48 hrs. after which it is tested for sterility. It is then transferred in 0.25 ml quanities to ampoules and dried as in (2). The ampoules are sealed, and stored at 4°C. Sterility tests show that these procedures effectively prevent the survival of *S. typhi* and other bacteria.

(d) *Reconstitution of vaccine*

Immediately before use the vaccines are suspended in distilled water to concentration of 10^9 organisms/ml.

(e) *Mouse protection tests*

Vaccines produced by the foregoing procedures have been compared with the WHO reference vaccine K, and with Ty2A vaccine (both of which were prepared from Ty2, the motile ancestor of *S. typhi* TNM1) by the following procedure.

Female mice of the Theiler Original strain were used. The average weight was 15 to 16 g at vaccination, and 18 to 19 g at the time of challenge. In each test 3 doses of each vaccine were used: 5×10^8, 5×10^7, 5×10^6 organisms. 16 mice were injected with each dose. Each mouse received one dose of vaccine intraperitoneally in a volume of 0.5 ml., the challenge being administered 7 days after inoculation. The challenge organism was strain Ty2 of *S. typhi*, grown for 6 hr. on nutrient agar at 37°C and washed off with 0.85% saline. The LD50 of the challenge strain, administered intraperitoneally in 5% mucin, was 2.7×10^3 to 6.9×10^3 organisms. The challenge dose was 1.5×10^4 organisms administered intraperitoneally in a volume of 0.5 ml. in 5% mucin.

Four groups of mouse protection tests were performed. In the first two, vaccines Ty2A and TNM1A were compared with the WHO reference vaccine K. In the third and fourth groups vaccines TNM1B and TNM1PL were compared with TNM1A.

The results of the first and second tests for each vaccine were pooled, as were those of the third and fourth tests. The efficacies of the vaccines were calculated as ImD50, that is, the doses (in millions of organisms) needed to protect 50% of mice against death (which usually occurred within 48—72 hr.) when challenged with strain Ty2. Table 1 shows the results of these tests.

TABLE 1

		Vaccine				
		K	Ty2A	TNM1A	TNM1B	TNM1PL
Series 1	ImD50 (millions)	2.3	3.25	2.3		
Series 2	ImD50 (millions)			2.9	2.1	12.0

It is concluded that the acetone-killed and dried vaccine TNM1A and the alcohol-killed and dried vaccine TNM1B have a protective power equal to that of the vaccines prepared from the ancestral motile strain. The heat-killed phenolized vaccine appears to have somewhat lower protective value in mice than the other vaccines tested.

EXAMPLE 2

The procedures described in Example 1 from section (b) to (d) are followed, employing *S. paratyphi* A strain NCTC10405 and separately for *S. paratyphi* B strain NCTC10406.

EXAMPLE 3

To examine the serological response in rabbits, a composite TAB vaccine containing *S. irigny* as an independent marker is prepared. The primary component of the vaccine is prepared substantially as described in Example 1. The paratyphoid organisms and the marker are grown separately on nutrient agar slopes and washed off with saline. The final vaccine is prepared by mixing the separately standardised suspensions to give final concentrations as follows:—

S. typhi TNM1	1.0×10^9/ml.
S. paratyphi A 17689	5.0×10^8/ml.
S. paratyphi B BB6981	5.0×10^8/ml.
S. irigny	2.0×10^8/ml.

Inoculation of rabbits with the separate vaccines and combined vaccine have been carried out as follows:—

(1) *With separate serotypes*

Inoculations were carried out at alternating 3 and 4 day intervals for 5 or 6 doses.

Dosage (ml. of vaccine): 0.1, 0.2, 0.5, 1.0, 1.0.

Sample bleedings were taken before the commencement of inoculation and immediately preceding each dose. Final bleedings were carried out 3 days after the sixth dose.

(2) *With composite vaccine*

Dosage (ml.): 0.2, 0.2, 0.5, 0.5, 1.0, 1.0

Results

(a) *Rabbits innoculated with separate serotypes*

Serotype	Agglutinins produced	
	O	H
S. paratyphi A 17689	AO 250	<25
S. paratyphi B BB6981	BO 1600	<25
S. irigny	*S. irigny* 0.70	*S. irigny* H 4,500
	AO <25	AH <25
	BO <25	TH <25
	TO <25	BH <25

The titres are given as reciprocals of the serum dilutions, and are geometric means of the responses in (usually) 3 rabbits. None of the non-motile strains produced H agglutinins.

S. paratyphi A 17689 produced A0 agglutinin at a mean titre of 0.

S. paratyphi B BB6981 produced a mean B0 titre of 1600.

S. irigny produced a mean z38 (H) titre of 4,500 and a doubtful 43 (0) titre of 70.

(b) *Rabbits innoculated with the composite "non-motile" vaccine containing S. irigny*

	Agglutinins		
	O	H	TVi
S. paratyphi A	500	<25	.
S. paratyphi B	800	<25	.
S. typhi	2000	<25	640
S. irigny	25	2000	.

Rabbits inoculated with the composite vaccine, therefore, produced 0 agglutinins for *S. paratyphi* A, *S. paratyphi* B, and *S. typhi*; Vi agglutin for *S. typhi*; and H agglutinin for *S. irigny* only. For human immunisation, the vaccine will contain the same amounts of organisms as given in this Example and grown in a similar manner but processed as described in Example 1 (c) and (d).

WHAT WE CLAIM IS:—

1. A killed anti-typhoid vaccine or anti-paratyphoid vaccine formulated for parenteral administration to humans in which the protective antigen is a stable non-motile strain of *Salmonella typhi* or *Salmonella paratyphi* A or B which is devoid of flagella capable of producing the corresponding TH, AH, or BH antibodies.

2. An anti-typhoid vaccine according to Claim 1, in which the strain of *S. typhi* is a genetically non-motile strain obtained by a process of selection employing a bacteriophage to eliminate motile organisms.

3. A vaccine according to Claim 2, in which the strain of *S. typhi* is strain N.C.T.C. 10404.

4. An anti-paratyphoid A or B vaccine according to Claim 1, in which the strains of *S. paratyphi* A or B employed are naturally non-motile strains.

5. An anti-paratyphoid A vaccine according to Claim 4 in which the strain employed is N.C.T.C. 10405.

6. An anti-paratyphoid B vaccine according to Claim 4, in which the strain employed is N.C.T.C. 10406.

7. A combined TAB vaccine, in which at least one of the protective antigens is as defined in any of the preceding claims.

8. A vaccine according to any of the preceding claims, containing an independent antigenic marker.

9. A vaccine according to Claim 8, in which the antigenic marker is killed *S. irigny*.

10. A combined TAB vaccine according to Claim 9 prepared from the following bacterial strains:—

S. typhi N.C.T.C. 10404
S. paratyphi A N.C.T.C. 10405
S. paratyphi B N.C.T.C. 10406
S. irigny N.C.T.C. 10407

11. A vaccine according to Claim 10, in which the organismal concentrations are, in the order given, 1.0×10^9/ml, 5.0×10^9/ml, 5.0×10^8/ml, and 2.0×10^8/ml.

12. A vaccine according to any of the preceding claims, being a heat-killed phenolized or an acetone-killed and dried or alcohol-killed and dried vaccine.

13. A process for the preparation of a killed anti-typhoid or anti-paratyphoid vaccine which comprises cultivating a stable non-motile strain of *Salmonella typhi* or *Salmonella paratyphi* A or B devoid of flagella capable of producing the corresponding TH, AH, or BH antibodies and thereafter processing the culture into the form of a killed vaccine suitable for parenteral administration to humans.

14. A process for the preparation of anti-typhoid vaccine according to Claim 13, in which the strain of *S. typhi* is a genetically non-motile strain obtained by a process of selection employing a bacteriophage to eliminate motile organisms.

15. A process according to Claim 14, in which the strain of *S.typhi* is strain N.C.T.C. 10404.

16. A process for the preparation of an anti-paratyphoid A or B vaccine according to Claim 13, in which the strains of S. paratyphi A or B employed are naturally non-motile strains.

17. A process according to claim 16, in which the strain of paratyphoid A or B

organism employed is N.C.T.C. 10405 or N.C.T.C. 10406 respectively.

18. A process for the preparation of a TAB vaccine, in which the strains are cultivated separately and then combined, at least one of the strains being as defined in any of the preceding claims.

19. A process according to any of Claims 13 to 18 in which an independent antigenic marker is incorporated in the vaccine.

24. A process according to Claim 19 in which the marked is killed *S.irigny*.

21. A process according to Claim 20 for preparing a TAB vaccine in which the organisms cultivated and then combined are

S.typhi	N.C.T.C. 10404
S.paratyphi A	N.C.T.C. 10405
S.paratyphi B	N.C.T.C. 10406
S.irigny	N.C.T.C. 10407

22. A process according to Claim 21, in which the vaccine is adjusted to a final organismal concentration, in the order given, of 1.0×10^9/ml, 5.0×10^8/ml, 5.0×10^8/ml, and 2.0×10^8/ml.

23. A process for the preparation of a vaccine, substantially as described in any of the Examples.

24. A vaccine when prepared by the process defined in Claim 23.

R. C. CRESPI,
Chartered Patent Agent,
Agent for the Applicants.

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